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RADIATION SYNTHESIS OF SILVER NANOPARTICLES/CHITOSAN AGAINST *Corynespora cassiicola* CAUSING LEAF FALL DISEASE ON RUBBER TREES

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ABSTRACT

In this study, silver nanoparticles (AgNPs) were prepared by gamma rays irradiation of 1.0, 2.5, 5.0 and 10 mM silver nitrate solution using chitosan as a stabilizer. UV spectra, morphology and size of AgNPs irradiated at different doses were characterized by using UV-vis spectrophotometer and TEM images. The obtained results indicated that the average size of AgNPs increased by the increase of silver concentration in irradiated solution or the degree of acetylation of chitosan, while the increase of chitosan concentration was found to be a functional key for reducing the average size of particles in AgNPs product. *In vitro* test, AgNPs inhibited the growth of *Corynespora cassiicola*. In particularly, the inhibitory efficiency of AgNPs on the growth of *C. cassiicola* on rubber leaf extract media increased from 52.1 to 100 % when the average particle size of particles in AgNPs product decreased from 15 to 5 nm at the concentration of 50 ppm. In addition, the increase of AgNPs concentration from 10 to 90 ppm also enhanced the antifungal activity to be from 6.3 to 100 %, respectively. It suggests that the silver nanoparticles/chitosan (AgNPs/chitosan) synthesized by γ-rays irradiation method is a very promising fungicidal product applying for treating *C. cassiicola*, a serious pathogen fungus on rubber trees.

Keywords: antifungal activity, chitosan, *Corynespora cassiicola*, gamma irradiation, leaf fall disease, silver nanoparticles.

1. INTRODUCTION

Rubbers (*Hevea brasiliensis*) are long day – industrial trees and provide raw materials for many industrial sectors. They possessed very high economic values and have already brought

many profits for Viet Nam agriculture. According to General Statistics Office of Viet Nam (2015), Viet Nam is now having the third ranking in total production (7.9 %), fourth in export (11.2 %) of natural rubber all over the world, just behind Thailand, Indonesia and Malaysia. However, rubber growers are now facing many difficulties due to the outspread of many diseases caused by microorganisms. Among which, leaf fall disease caused by *Corynespora cassiicola* are now severely affecting rubbers growth and yield [1]. This disease was first observed on rubber trees in Sierra Leone (Affrica, 1936). More cases were reported in India and Malaysia in 1961; Nigeria in 1968; Thailand, Srilanca and Indonesia in 1985; Brazil and Bangladesh in 1988 [2]. Though leaf fall disease in rubbers was only observed in Viet Nam from August 1999, the disease was spread rapidly and widely in many countries in Southeast, Central Highlands and Central Coast of Viet Nam. Prevention of leaf fall disease is now still a problem due to lack of specific fungicides while growers are now using many chemical derived products that would cause various negative impacts on the environment as well as rubber quality. Chitosan at low molecular weight has been proved as a natural, safe and effective product for agriculture [3]. Many researches have been already reported that besides having growth enhancing effects on plants, chitosan also provides plants with the ability to prevent many pathogenic infections by boosting the immunity system of plant cells - so called phytoalexin effects [4, 5, 6]. In addition, silver nanoparticles were extensively studied and widely used for a long time due to their unique properties such as antibacterial, fungal inhibition, odor removal, etc. at low concentration as well as safe to human and the environment. In Viet Nam, the antifungal effect of silver nanoparticles stabilized in chitosan has been studied on pathogen fungi such as *Phytophthora capsici* and *Corticium salmonicolor* [7, 8]. Moreover, irradiation with gamma rays using a Co-60 source was considered as an effective method of silver nanoparticles synthesis. The advantages of irradiation method were energy, room and materials saving; environmental friendly. The process can be conducted at ambient temperature and could be easily up-scaled to pilot production with reasonable price [9, 10, 11]. This research aimed to synthesize silver nanoparticles/chitosan from natural product, safe to human and effective in elimination of leaf fall disease caused by *C. capssiicola* on rubber trees.

2. MATERIALS AND METHODS

2.1. Materials

Pure silver nitrate powder ($AgNO_3$) was obtained from Merck, Germany. Chitosan at different degree of deacetylation of 70 (7B), 80 (8B), 90 (9B) and 99.9 % (10B) were purchased from Loyou Chemical Co. Ltd, Japan. Pathogenesis fungus namely *Corynespora cassiicola* was a gift from Rubber Research Institute of Viet Nam.

2.2. Synthesis of AgNPs/chitosan using γ Co-60 irradiation

Silver nitrate solution at different concentration of [Ag^+] 1.0; 2.5; 5.0 and 10 mM stabilized in chitosan solution of 1, 2, 3 and 5 %. These samples were stored in glass bottles and irradiated at different doses from 4 to 28 kGy using γ-rays with a dose rate of 3 kGy/h from a Co-60 BRIT 5000 source (India), Dalat Nuclear Research Institute. Besides that, silver nitrate solutions at 10 mM [Ag^+] stabilized in chitosan with different degree of deacetylation (7B, 8B, 9B and 10B) were also irradiated at 20 kGy. Silver nanoparticles/chitosan samples synthesized by gamma irradiation were used for further experiments.

2.3. Evaluation of silver nanoparticles/chitosan properties

The stability of irradiated AgNPs/chitosan was evaluated by UV-vis spectroscopy. AgNPs samples were diluted to 0.1 mM of [Ag^+] using deionized water. UV-vis spectroscopy was measured using UV-Vis (UV-2401PC Shimadzu, Japan) spectrophotometer [10, 12]. Silver nanoparticles size and distribution were determined by TEM imaging using JEM 1400, JEOL (Japan) followed Li et al. method [10].

2.4. Antifungal activity of AgNPs/chitosan

Rubber-leaf extract media was used for *C. cassiicola* culture. One liter of rubber leaf extract (at pH 6.5) was mixed with 20 g agar and supplemented with AgNPs/chitosan at different particle sizes 5; 10 and 15 nm with AgNPs concentration varied from 0 to 90 ppm. AgNPs with particle size of 5 and 10 nm were synthesized using irradiation method from silver nitrate solutions at pH 6 with the concentrations of [Ag^+] were 1 and 10 mM, respectively. AgNPs with particle size of 15 nm were obtained from silver nitrate in chitosan solution at pH 3 and 5 mM of [Ag^+]. Fungal samples with diameter ~1 mm were culture on the center of rubber-leaf extract agar plates and incubated in dark at 28 ± 2 $^{\circ}$C. Five replicate plates were applied for each concentration or particle size text and all experiments were repeated in triplicate. The colony diameters of *C. cassiicola* in the medium were measured each 24 hours and the antifungal efficiency of AgNPs/chitosan was calculated using the following equation:

$$\text{Inhibition (\%)} = (D\text{-}d/D) \times 100$$

where D and d (mm) are fungal colony diameters on the medium with or without supplemented with AgNPs/chitosan, respectively.

3. RESULTS AND DISCUSSION

3.1. Saturated conversion dose determination of silver nanoparticles/chitosan

The saturated conversion dose is the radiation dose at which [Ag^+] converses totally into Ag and the UV-vis absorbance reaches maximum. In this experiment, chitosan concentration was used at 1 % and the results from Figure 1 and Table 1 indicated that the saturated conversion doses were 8, 12, 16 and 20 kGy corresponded to the [Ag^+] concentrations of 1; 2.5; 5 and 10 mM, respectively. The maximum absorbance wavelength increased from 397 to 405.5 nm by the increase of [Ag^+] concentration. In addition, AgNPs size measured using TEM imaging (Figure 2) were 5.18; 7.13; 8.69 and 10.06 nm corresponded to [Ag^+] solution of 1; 2,5; 5 and 10 mM. These results indicated that the concentration of [Ag^+] affect the size of silver nanoparticles formed in the irradiated samples and the increase of [Ag^+] concentration led to the increase of nanoparticle size.

Besides that, many researches have been already proved that the germicidal activities of silver nanoparticles increased by the decrease of particle size [7, 13]. Thus, in order to synthesize silver nanoparticles with desired size for different applications, [Ag^+] concentration in the initial samples is very important.

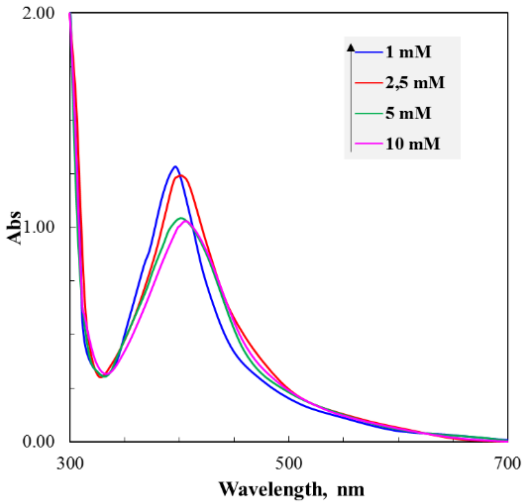

Figure 1. UV-vis spectroscopy of silver nanoparticles/chitosan at different [Ag^+] concentration.

Table 1. Properties of irradiated silver nanoparticles/chitosan.

[Ag^+] concentration, mM	λ_{max}, nm	OD	Particle size, nm
1	397.5	1.26	5.18 ± 0.18
2.5	402.5	1.20	7.13 ± 0.34
5	403	1.03	8.69 ± 0.5
10	405.5	1.02	10.06 ± 0.57

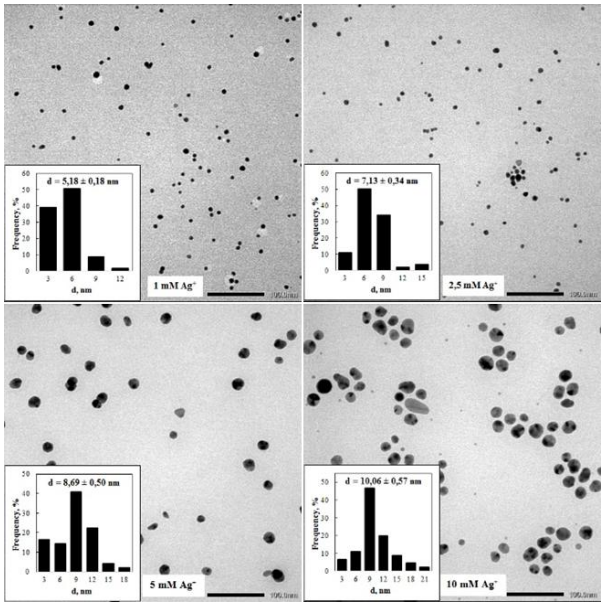

Figure 2. TEM images and size distributions of silver nanoparticles at different [Ag^+] concentration.

3.2. Influence of chitosan concentration to the size of the nanoparticles

Huang et al. [14] reported that different silver nanoparticle sizes would lead to various maximum wavelength absorbance and peak intensities. AgNPs size increased, their λ_{max} shifted to the longer wavelength [15, 16]. Results from Table 2 indicated that λ_{max} of AgNPs synthesized from silver nitrate solution of 10 mM [Ag^{+}] stabilized in chitosan of 1, 2, 3 and 5 % varied from 399.5 to 405.5 nm, corresponded to AgNPs size (Figure 3) 10.06; 8.6; 7.84 and 6.04 nm respectively. Thus, the increase of chitosan concentration in irradiated samples led to the decrease in particle size of silver nanoparticles synthesized and the same results were also reported by Yoksan et al. [17]. In addition, results from Figure 3 also showed that the size distributions of AgNPs became narrower when the chitosan concentration increased. Though the mechanics of these effects was not yet fully understood, the increase of solution viscosity corresponded to the increase of chitosan concentration might have reduced the dynamics of AgNPs synthesized and prevented them from forming bigger nanocluster.

Table 2. Properties of irradiated AgNPs/chitosan stabilized in chitosan at different concentration.

Chitosan concentration, %	λ_{max}, nm	OD	Particle size, nm
1	405.5	1.02	10.06 ± 0.57
2	403.0	1.03	8.60 ± 0.25
3	402.5	1.01	7.83 ± 0.27
5	399.0	1.11	6.40 ± 0.26

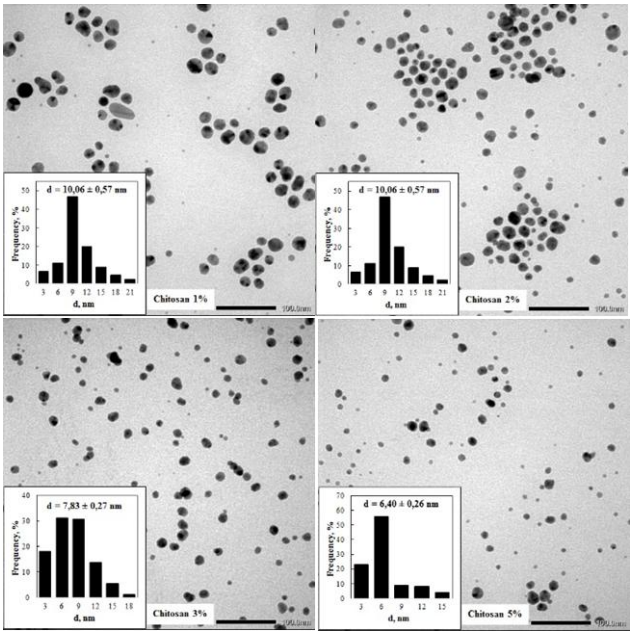

Figure 3. TEM images and size distributions of silver nanoparticles stabilized in chitosan at different concentration.

3.3. Influence of chitosan degree of acetylation (DDA) to nanoparticles size

Results from Table 3 showed that DDA of chitosan also affected AgNPs properties. Maximum absorbance wavelength (λ_{max}) of silver nanoparticles varied from 402.5 to 407 nm. In addition, nanoparticle size measured using TEM imaging (Figure 4) also indicated that higher DDA led to bigger particle size. The AgNPs size were 8.67, 10.06, 10.78 and 11.88 nm corresponded to the DDA of 70, 80, 90 and 100 %. The results could be explained by the decrease in size of chitosan molecular when acetyl (-$COCH_3$) groups were removed, which led to the increase of silver nanoparticles dynamics and boosted the rate of nanocluster formation. However, the reason of these events has not yet been explained by any research.

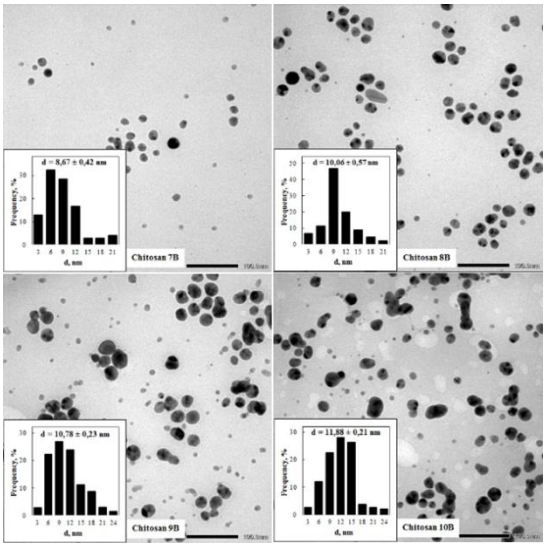

Figure 4. TEM images and size distributions of AgNPs stabilized in chitosan at different DDA.

Table 3. Properties of irradiated silver nanoparticles/chitosan stabilized in chitosan at different DDA.

DDA, %	λ_{max}, nm	OD	Particle size, nm
70	402.5	0.98	8.67 ± 0.42
80	405.5	1.02	10.06 ± 0.57
90	406.5	1.00	10.78 ± 0.23
100	406.5	0.84	11.88 ± 0.21

3.4. Antifungal activities of AgNPs/chitosan against *C. cassiicola*

3.4.1. Influence of AgNPs concentration on C. cassiicola inhibition

Many previous researches have already proved that antifungal activities of AgNPs increased by the increase of nanoparticles concentration [18, 19, 20]. In this research, AgNPs/chitosan synthesized from solution of 10 mM [Ag^+] and 1 % of chitosan 8B were used to evaluate the antifungal activities against *C. capssiicola* on rubber-leaf extract agar at different AgNPs concentrations. Results from Tables 4 indicated that *C. capssiicola* growth normally on control plates with the fungal growth diameter reached 90 mm after 10 days of incubation (Figure 5). Meanwhile, on the agar plates supplemented with AgNPs at 10 nm particle size and concentration from 10 to 30 ppm, the growth inhibition against *C. capssiicola* has not yet been

observed. However, the growth of *C. capssiicola* was clearly inhibited when the concentration of AgNPs/chitosan increased to 50 ppm, with the fungal growth diameter only 50 mm after 10 days of incubation. The inhibition properties of AgNPs increased when AgNPs/chitosan concentration increased to 70 ppm and the growth of *C. capssiicola* was completely inhibited when the silver concentration reached 90 ppm.

Table 4. C. cassiicola inhibition effects of silver nanoparticles at different concentrations after 216 hours of incubation.

Silver nanoparticles concentration, ppm	Fungal colony diameter, mm	Inhibition, %
0 (Control)	90.00[a]	0.00[f]
10	84.33[b]	6.29[e]
30	73.33[c]	18.52[d]
50	30.25[d]	66.39[c]
70	7.50[e]	91.67[b]
90	0.00[f]	100.00[a]

Means values followed by the same letter within a column are not statistically different according to a Duncan's multiple ($P < 0.01$).

After 216 hours of incubation, antifungal activities of AgNPs at different concentrations of 10, 30, 50, 70 and 90 ppm against *C. capssiicola* were found at 6.29; 18.52; 66.39; 91.67 and 100 %, respectively (Tables 4). These results indicated that the antifungal activities of AgNPs product remained low when supplemented with AgNPs from 10 to 30 ppm. However, the fungal inhibition effect of the tested product increased rapidly when the supplemented AgNPs increased to 70 ppm, and the fungal growth was inhibited strongly (91.67 %) after 216 hours of incubation. Moreover, the antifungal efficiency was 100 % when the concentration of AgNPs reached 90 ppm (Figure 4 and Table 5). Thus, AgNPs have the inhibition effect on *C. cassiicola* growth and the antifungal efficiency increases by the increase of supplemented AgNPs in the culture media. The optimal antifungal efficiency against *C. cassiicola* was determined from 91.67 – 100 % by the treatments of AgNPs concentrations from 70 – 90 ppm.

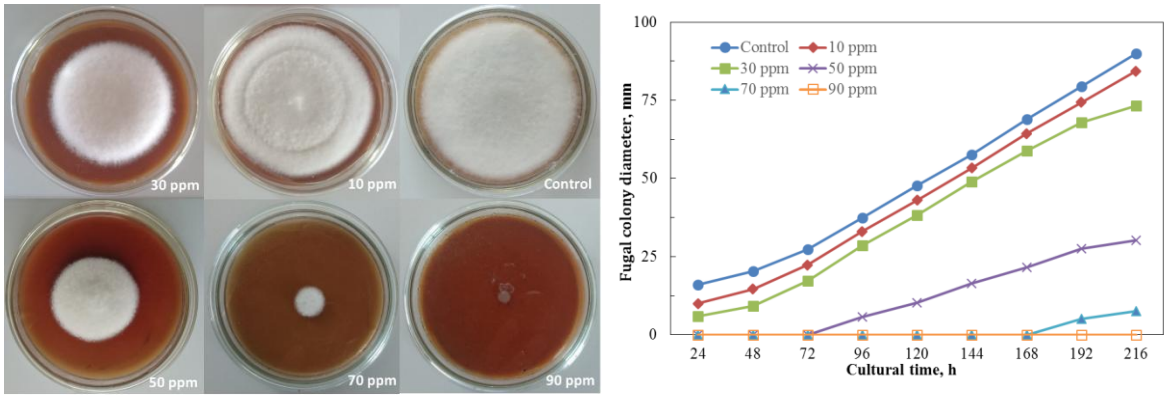

Figure 5. The growth of *C. cassiicola* after 216 hours of incubation on rubber-leaf extract media supplemented with AgNPs (10nm) at different concentrations.

3.4.2. Antifungal efficiency of AgNPs /chitosan at different particle sizes

Silver nanoparticles with particles size at 5, 10 and 15 nm were selected for antifungal efficiency testing. Results from Figure 6 and Table 5 indicated that the antifungal efficiency increased strongly when the particle size decreased. After 192 hours of incubation, *C. capsiicola* growth normally on the control plates with the diameter reached 90 mm. However, cultural media supplemented with AgNPs at 50 ppm and 15 nm particles size showed strong inhibition effects on *C. capsiicola* with the fungal diameter only reached 43.13 mm (52.08 % inhibition). The antifungal efficiency increased to 70.56 % when the particle size decreased to 10 nm (Tables 5) and reached to 100 % at 5 nm particle size (Figure 6).

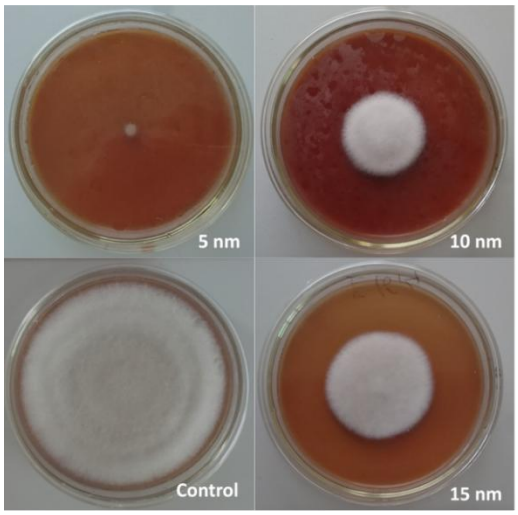

Figure 6. The growth of *C. capssiicola* after 192 hours of incubation on rubber-leaf extract media supplemented by silver nanoparticles/chitosan with different particle sizes.

Table 5. Antifungal efficiency against *C. cassiicola* of silver nanoparticles at different particle sizes after 192 hours of incubation.

Particle size, nm	Fungal colony diameter, mm	Inhibition, %
Control	90.00[a]	0.00[d]
5	0.00[d]	100.00[a]
10	34.00[c]	62.22[b]
15	45.50[b]	49.44[c]

Mean values followed by the same letter within a column are not statistically different according to a Duncan's multiple text ($P < 0.01$).

Thus, the antifungal efficiency increased by the decrease of AgNPs size. These results were also reported in previous researches of Le et al. [7] and Carlson et al. [13]. In addition, Franci et al. [21] also pointed out that it is easier for AgNPs at smaller size to penetrate through cell walls, alter and inhibit the cell signaling pathways as well as DNA replication and damage cell organs through oxidative reaction.

4. CONCLUSION

Silver nanoparticles with different particle sizes were successfully synthesized by gamma Co-60 irradiation method using chitosans at different concentrations and degrees of deacetylation as stabilizers. The synthesized AgNPs/chitosan products showed a strong inhibition effect against *C. capssiicola* with the antifungal efficiency reached 100 % by the treatment with 50 ppm AgNPs at the particle size of 5 nm or 90 ppm AgNPs at the particle size of 10 nm. Thus, AgNPs/chitosan synthesized using irradiation method could be a potential product for treatment of *Corynespora* leaf fall disease on rubber trees.

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